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ISOLATION OF CALDATRIBACTERIUM (OP9) AND INVESTIGATION OF ITS POTENTIAL INTERACTIONS WITH A NOVEL, CO-CULTIVATED THERMODESULFOBACTERIUM SPECIES

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ISOLATION OF CALDATRIBACTERIUM (OP9) AND INVESTIGATION OF ITS POTENTIAL INTERACTIONS WITH A NOVEL, CO-CULTIVATED *THERMODESULFOBACTERIUM* SPECIES

A Thesis

Presented to the

Faculty of

California State University,

San Bernardino

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in

Biology

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by

Toshio Alvarado

September 2019

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ABSTRACT

Atribacteria (OP9), candidate phylum with no representatives in pure culture, is found in various anaerobic environments worldwide. “Caldatribacterium”, a lineage within Atribacteria that is predicted to be a strictly anaerobic sugar fermenter based on cultivation-independent genomic analyses, is currently being maintained in lab enrichment cultures with fucose as its sole growth substrate. Metagenomics and 16S rRNA gene tag sequencing indicated that the fucose culture was a co-culture of “Caldatribacterium” and an uncultivated member of the genus Thermodesulfobacterium. Due to failed attempts to isolate “Caldatribacterium” by dilution-to-extinction and plating, it was hypothesized that “Caldatribacterium” is dependent in some way on the Thermodesulfobacterium. To better understand the possible interaction, multiple isolates of the sulfate reducer were obtained under sulfate-reducing conditions with H_2 as an electron donor, and one of the isolates was characterized. Whole genome and 16S rRNA gene sequence comparisons of the isolate and other related members of the genus *Thermodesulfobacterium* suggested the isolate represents a distinct species in this genus, for which the name *T. auxiliatoris* is proposed. *T. auxiliatoris* was capable of using H_2, formate, and lactate as sole electron donors, but not fucose or other sugars, suggesting that its growth in the co-culture might be dependent on one or more fermentation substrates produced by “Caldatribacterium”. Addition of *T. auxiliatoris* to highly diluted samples of the co-culture that likely contained only “Caldatribacterium”, which did not exhibit

growth on their own, demonstrated that *T. auxiliatoris* was sufficient to support growth of “Caldatribacterium” on fucose. When this dilution experiment was repeated with various other organisms and substrates, it was found that several other thermophilic sulfate reducers (*T. commune*, *T. hveragerdense*, or *Thermodesulfovibrio yellowstonii*) could also support growth, as well as supernatant from the *T. auxiliatoris* pure culture or yeast extract. This last finding allowed for isolation of “Caldatribacterium”, which could form colonies on solid media when yeast extract and casamino acids were present. Fluorescent *in situ* hybridization and nanometer-scale secondary ion mass spectrometry demonstrated that “Caldatribacterium” took up a variety of sugars and amino acids in mixed culture, and that addition of acetate or bicarbonate, substrates of *T. auxiliatoris*, stimulated sugar uptake in “Caldatribacterium”. These results support a model where *T. auxiliatoris* and “Caldateribacterium” are dependent on each other in co-culture on fucose, where “Caldatribacterium” provides growth substrates for *T. auxiliatoris*, which in turn provides “Caldatribacterium” with some sort of soluble, essential compound(s) that can be produced by other sulfate reducers and are present in yeast extract. Further characterization of the “Caldatribacterium” isolate, the first representative of the phylum Atribacteria, will allow for detailed study of its metabolic capabilities that can be extended to other members of this phylum. Further analysis of responses of *T. auxiliatoris* and “Caldatribacteirum” when grow in co-culture and the specific metabolite(s) that

are exchanged between the two organisms could allow for testing whether these interactions occur in more complex, natural systems.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to my committee chair, Dr. Jeremy Dodsworth, who accepted me into his lab in fall quarter 2015. Since then, he has taught me many valuable skills and has helped me grow as a person. Without his guidance this would have not been possible.

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CHAPTER ONE

INTRODUCTION

Uncultivated Microbes

From the initial observation of Bacteria by Antonie van Leeuwenhoek to the pioneering work in obtaining pure cultures of microbes by Pasteur, Koch, and others that ushered in the “golden age” of microbiology, the bulk of our knowledge of microorganisms is based on studying them in pure culture in the laboratory. However, the size and diversity of the microbial community has not been fully appreciated until recent times. Microorganisms make up a large part of Earth’s biomass and are found in many environments due to their importance in primary production (Whitman et al., 1998). More recent observations like the “great plate count anomaly”, suggested to microbiologists that many microbes found in various environments have yet to be cultured (Staley & Konopka, 1985). We now know that most microbes have yet to be studied in pure culture (Whitman et al. 1998). However, in recent years advances in DNA sequencing and cultivation-independent approaches have greatly enhanced the ability to study yet-uncultivated microbes. These techniques have given the scientific community a greater understanding of the diversity and physiological potential of the microbial world, and yet-uncultivated microbes have been collectively referred to as “Microbial dark matter” (Hedlund et al., 2014). The phrase “dark matter” is borrowed from the field of astronomy where it is used to highlight the fact that most mass in the universe is not observable using standard techniques.

In microbiology, “Dark matter” is known to exist at all taxonomic levels, from species to phylum.

Candidate Phyla

A candidate phylum is a phylum-level lineage with no representatives in pure culture. Currently is it estimated that there could be around 1,500 bacterial phyla and 300 archaeal phyla, based on an analysis of 16S rRNA gene sequences in the SILVA REF 114 database (Yarza et al., 2014). Currently there are only cultivated representatives for approximately 33 of these, making the vast majority candidate phyla. For many of these candidate phyla, little is known besides their existence from analyzing their 16S ribosomal-RNA gene sequences. Although cultivation-independent genomic methods have allowed for prediction of the physiology of members of some candidate phyla (Rinke et al., 2013; Hedlund et al., 2014), many of these predictions remain to be tested. In order to better characterize uncultivated microbes, it is very useful to be able to study them in pure or mixed culture in a laboratory setting (Stewart, 2012). However, laboratory cultivation of many of these microbes can be challenging.

Difficulty of Growing Microbes in Lab

Most of what we know about microbes to date has been learned by studying them in pure culture. There are other relatively new techniques like metagenomics that can predict certain function, but pure culture will remain a staple in microbiology. Therefore, understanding why uncultivated microbes are

difficult to cultivate is important in trying to cultivate and study them. Difficulty in cultivation is largely due to the inability to mimic the precise environmental components necessary for survival such as temperature, growth factors, pH, buildup of waste products, and syntrophic interactions with other microbes. Depending on the microbe it may or may not need every component from its environment, but just particular aspects from it (Stewart, 2012). However, finding which components are necessary for cultures to grow in lab has proven to be difficult. This is not just limited to unknown components, but it can also include symbiotic relationships and/or unique environments.

Syntrophy is a relationship between two organisms that depend on one another in order to survive. The word “syntrophy” dates to the mid-twentieth century and was originally used to represent cross-feeding interactions (Morris et al., 2013). We now know that syntrophic relationships can span from mutualistic relationships where both partners benefit, in contrast to parasitism where one member benefits and the other is harmed. In many cases it is a service-type relationship, with one partner providing a compound that is consumed by the other in return for a reward (Bronstein, 1994). There are some opinions that this definition should be restricted to a dependence that cannot be separated even when a substrate is added as substitution for its partner (Schink, 1997), however the term syntropy has been used in other contexts where at least one of the syntrophic partners can be grown in pure culture under certain conditions. In contrast, Hillesland and Stahl (2010) studied the evolution of mutualism by taking

two microbes with no previously known interaction. They established a syntrophic relationship between a hydrogen-producing sulfate reducer and a hydrogen-consuming methanogen by creating a co-culture which allowed them to trade byproducts in the absence of sulfate. A standard example of syntrophy is observed in cultures of “Methanobacillus omelianskii”, which was once thought to be one archaeon but was subsequently shown to be a co-culture of two species of microbes in a syntrophic relationship (Barker 1936; Bryant et al. 1967). The benefit of a syntrophic relationship allows the organisms to grow more efficiently, often involving consumption of the waste products of a fermenter, such as hydrogen or organic acids, by a second organism under anaerobic conditions (Morris et al., 2013). Because of this, cultivation of syntrophic partners on their own, especially the fermenter in the partnership, can be challenging.

Only a few examples of syntrophy have been observed in thermophiles. One example is between two hyperthermophilic archaea, *P. furiosus* and *Methanopyrus kandleri* (Morris et al., 2013). When the two archaea are cultured together, they achieve a higher cell density compared to when partnered with other microbes and when they are grown in isolation. *P. furiosus* is known to ferment organic compounds and *M. kandleri* performs methanogenesis which suggests a syntrophic relationship based on hydrogen removal (Schopf et al., 2008). Although this example provides some insight to what may be happening during syntrophic relationships, little is known about the interaction between various microbes and what other factors are involved (Morris et al., 2013).

Unique environments can also play a factor in the ability to study uncultivated microbes. Although technology and cultivation techniques have improved in recent years, there are still difficulties in cultivating microbes from unique environments. For example, studying deep-sea microbes is challenging because of transitioning the microbes from their normal hydrostatic pressure from the bottom of the sea to surface pressures (Zhang et al. 2017). This does not include the challenge of accessing the microbes at such depths, maintaining an appropriate environment in lab, and other factors like types of nutrients mentioned above.

Recreating an environment for an unknown microbe is often difficult and time consuming, but some promising novel approaches have been developed. In 2002 researchers created a diffusion chamber that allows for uptake of nutrients from the environment but prevents the uptake of microbes with a semi-permeable chamber (Kaeberlein, 2002). This approach can be used in lab to maintain previously uncultivable microbes. Although this does not factor in possible syntrophic relationships, it provides an approach to culture microbes that might be dependent on exchange of small, soluble factors. Co-cultures and host-associated environments involve two or more bacteria maintained in the same sample. For many species of microbes, relationships are created in order to decrease the rate of energy expended under a given set of growth conditions (Morris et al., 2013). In some syntrophic relationships, neither microbe can

survive without its partner (Morris et al., 2013). However, when studying uncultivated microbes these relationships are unknown.

Methods for Studying Uncultivated Microbes

Because cultivation of new microbes continues to be challenging, microbiologists have turned to cultivation-independent techniques to learn about yet-uncultivated microbes. With the use of metagenomics and single-cell genomic sequencing, scientists can analyze their genome without maintaining cultures in lab (Hedlund et. al 2014). Metagenomic sequencing involves shotgun sequencing of DNA from an environmental sample containing a mix of microbes. These sequence fragments can be assembled into contigs, and contigs can be “binned” or assigned to individual organisms using various techniques, such as nucleotide frequency and sequencing coverage (Hedlund et. al 2014). This strategy is effective because it allows for studies of genomic content of microbes from essentially any environment. Single-cell genomic sequencing is taking a single cell and extracting its DNA for amplification and sequencing. The benefit of this method is it allows for a single genome to be analyzed, but isolation of single cells and subsequent extraction, amplification and assembly of entire genomes can difficult due to sensitivity to contamination and bias in amplification techniques (Hedlund et. al 2014). These methods allow for a greater understanding of the unknown microbes being studied, providing predictions of the metabolism of microbes that can potentially aid in their cultivation.

Another technique commonly applied to study yet-uncultivated microbes is fluorescence *in situ* hybridization (FISH), which can allow for identification of a specific type of microbe in a mixed sample (Amann and Fuchs, 2008). This technique involves hybridization of fluorescently labeled oligonucleotide probes to nucleic acid targets in fixed cells, allowing them to be visualized by fluorescence microscopy. The attachment site for the probes can be either DNA or RNA, but in unknown microbes the 16S rRNA is commonly targeted because it contains both conserved and variable regions. Probes targeting conserved regions can allow for detection of broad taxonomic groups, e.g. all Bacteria, while probes targeting variable regions can potentially identify specific genera or species of microbes. The first step to FISH is fixation, which is done to maintain the morphology and components of the cell. The probes used in FISH are bound specifically depending on formamide concentration. In PCR different temperatures are used to achieve appropriate specificity of primers. The same concept is applied here, however the formamide concentration is changed to optimize each specific type of probe at a fixed temperature. Use of the correct formamide concentration will allow the probes to bind specifically to their target. Once hybridization is completed the cells are washed and visualized under the fluorescent microscope.

In science, positive and negative controls are often used in experiments to validate and aid in interpretation of data, and to troubleshoot experiments. However, for FISH results to be valid for an uncultivated microbe, positive

controls must be created. The method used is Clone-FISH (Schramm et al., 2002), which allows for a pseudo-positive control to be created. The first step in Clone-FISH is retrieving the 16S rRNA sequence from a natural sample by amplifying it using PCR. Once amplified, the 16S rRNA gene can be ligated into a plasmid cloning vector. The plasmid can then be transformed into an appropriate host strain like E. coli. Expression of the heterologous 16S rRNA from the insert on the plasmid is induced by addition of IPTG and chloramphenicol. Once this is completed the cells can be fixed and hybridized with probes specific to the insert.

Atribacteria (OP9) and Laboratory Cultivation

Atribacteria (OP9) is an example of candidate phylum within the domain Bacteria, with no cultivated representatives currently in pure culture. Sequences corresponding to Atribacteria were originally discovered in Obsidian Pool, a hot spring in Yellowstone National Park, along with 11 other novel lineages (OP1-OP12) (Hugenholtz et al., 1998). Based on 16S rRNA gene surveys, members of the Atribacteria have been found in hot spring worldwide, but they have also been detected in a variety of other environments including marine sediments, petroleum reservoirs, digesters, and wastewater sludge treatment plants (Dodsworth et al. 2013). All the environments that Atribacteria have been found into date are anaerobic, suggesting that members of this candidate phylum are anaerobes. Recently, several genomes of members of the Atribacteria have been obtained using single-cell genomics and metagenomics techniques (Dodsworth

et al., 2013; Rinke et al., 2013; Nobu et al., 2015). In general, they are predicted to be strict anaerobes that carry out fermentation (Nobu et al., 2015). Atribacteria in hot springs, including the candidate genus-level lineage “Caldatribacterium”, are specifically predicted to be able to ferment a variety of sugar substrates, and may produce hydrogen, ethanol, acetate, and possibly other organic acids as products of fermentation (Dodsworth et al., 2013). They may also be capable of degrading carbohydrate polymers such as xylan and xyloglucan (Figure 1).

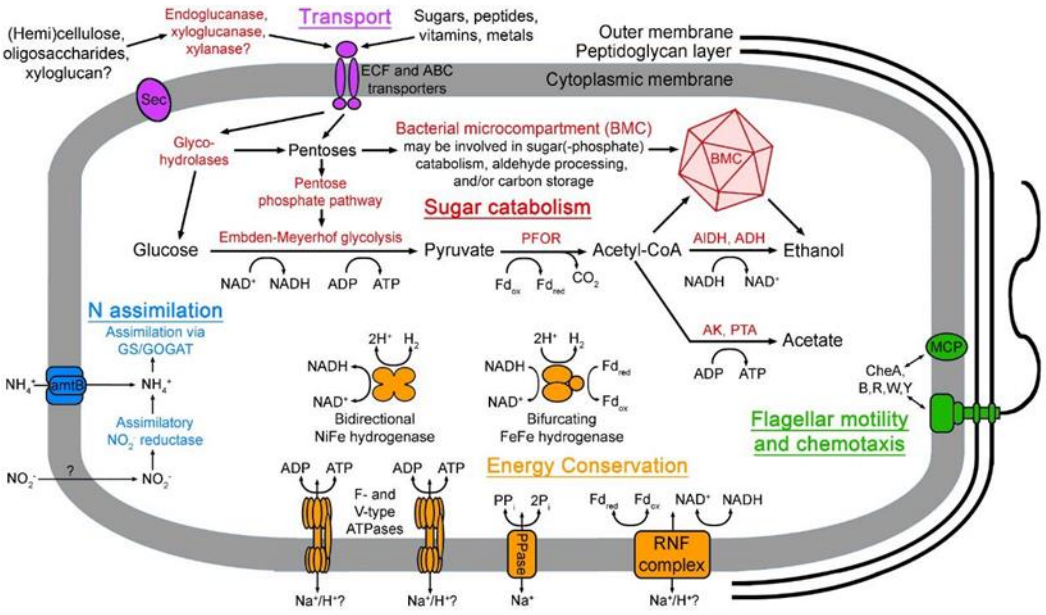

Figure 1. Predicted Cell Structure and Metabolic Pathways in “Caldatribacterium” (Dodsworth et al., 2013)

Consistent with these predictions, “Caldatribacterium” was found to be abundant in thermophilic consortia colonizing corn stover and aspen shavings incubated at ~75 °C in Great Boiling Spring, Nevada (Peacock et al., 2013). For the remainder of this proposal I will be referring to “Caldatribacertium” as OP9.

This thesis project revolves around study of a laboratory culture containing OP9 that was obtained from Great Boiling Spring (GBS). Figure 2 shows the method of obtaining the original mixed culture containing OP9, the methods of obtaining highly enriched cultures of OP9, and molecular methods used to assess the community composition of these cultures.

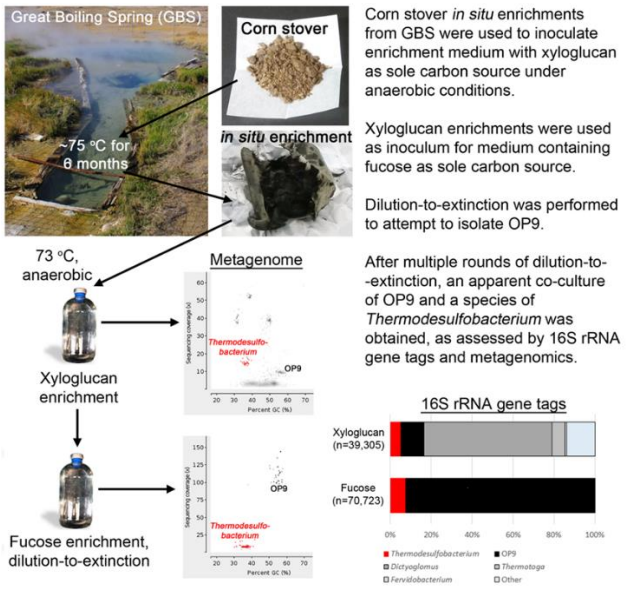

Figure 2. Xyloglucan- and Fucose-Degrading Enrichment Cultures containing OP9 and *Thermodesulfobacterium* were Derived from Corn Stover Enrichments in Great Boiling Spring, NV. Metagenomic Reads (Illumina MiSeq, 2x250) were Assembled and Binned using MetaWatt (Strous et al., 2012). 16S rRNA Gene tag Amplification and Sequencing was Performed as described in Kozich et al. (2013).

To attempt to cultivate OP9, *in situ* corn stover enrichments in GBS were used to inoculate an enrichment medium with xyloglucan as the sole carbon source. In the laboratory, the culture was incubated at 73˚C and transferred to new medium every two weeks. OP9 was detected in the culture by PCR, qPCR, and FISH. The abundance of OP9 in these xyloglucan enrichments was on average 5% based on the techniques used. Dr. Dodsworth and members of his lab tested multiple substrates in order further enrich for OP9, using the xyloglucan cultures as inoculum. Identifying which substrates allowed for enrichment of OP9 was done using PCR, qPCR, and FISH. It was found that OP9 was significantly enriched when grown on several different sugars as individual substrates (but not complex carbon sources like yeast extract, peptone, or casamino acids), with the highest relative abundance of OP9 of 50% using the substrate fucose. To further enrich for OP9, dilution-to-extinction experiments were performed on these fucose cultures. In in order to analyze abundance qPCR and FISH was used. The FISH experiments required optimizing the OP9 probes which was found to be 30% formamide. Based on qPCR and FISH, it was found that OP9 was highly enriched (95%) after repeated dilution-to-extinction. To continue studies of the OP9 culture, the other microbes present in the culture were identified using 16S rRNA gene sequencing and metagenomics. It was found that one other bacterium was with OP9 as a co-culture, a species within the genus Thermodesulfobacterium. Experiments conducted to separate OP9 and the *Thermodesulfobacterium* species by dilution-

to-extinction and plating were successful, leading to the hypothesis that OP9 depends on the *Thermodesulfobacterium* in the fucose co-cultures, possibly in a syntrophic relationship.

Recently, a collaborator of Dr. Dodsworth, Dr. Scott Hamilton-Brehm at Southern Illinois University at Carbondale (SIUC), obtained an enrichment culture containing a relative of OP9 (~98% 16S rRNA gene identity to the OP9 in the fucose enrichment metagenome). This enrichment culture, growing on xylitol as a major carbon source, was derived from a sample taken from a deep-subsurface (~500 m below ground) borehole water sample in southern Nevada. Although the original sample appeared to be dominated by other microbes, 16S rRNA gene tag sequencing and metagenomics performed by Drs. Hamilton-Brehm and Dodsworth on DNA extracts from this enrichment suggested that it too represented a co-culture of this OP9 relative (OP9_SIUC) and a sulfate reducer in the genus *Thermodesulfovibrio*, with the latter being only ~2% of the sequencing reads. Dr. Dodsworth was successful in isolating a sulfate reducer from this culture that is very closely related (99.8% 16S rRNA gene identity) to *Thermodesulfovibrio yellowstonii*, a member of the phylum Nitrospirae. Repeated attempts to isolate the OP9 relative from the *Thermodesulfovibrio* in the SIUC culture by dilution-to-extinction were not successful, suggesting that this related OP9 species may have a dependence on a sulfate reducer that is similar to the apparent dependence of OP9 on *Thermodesulfobacterium* in the fucose enrichments.

Sulfate Reducers and the Genus *Thermodesulfobacterium*

Thermodesulfobacterium is a genus in the phylum *Thermodesulfobacteria* containing sulfate reducing species. Like most other sulfate reducers, they are strict anaerobes that can use sulfate as a terminal electron acceptor in anaerobic respiration. Currently this genus contains four published distinct species, *Thermodesulfobacterium* geofontis (Hamilton-Brehm et al., 2013), *Thermodesulfobacterium* commune (Zeikus et al., 1982), *Thermodesulfobacterium* hydrogeniphilum (Jeanthon et al., 2002), and *Thermodesulfobacterium* hveragerdense (Sonne-Hansen & Ahring, 1999). Characteristics of the four species are seen in Table 1 (OPF15, 4, 5, and 6). All four related species use sulfate as an electron acceptor. Other electron acceptors utilized by at least some *Thermodesulfobacterium* isolates are sulfite, thiosulfate, and elemental sulfur. Electron donors used can include hydrogen, formate, lactate, and pyruvate, although most species can only use a subset of these. *Thermodesulfovibrio yellowstonii*, although it is a member of a different phylum (Nitrospirae), is phenotypically similar to members of the species *Thermodesulfobacterium* (column 3 in Table 1). Since the members of this genus use these compounds, it suggests that the *Thermodesulfobacterium* in the fucose culture with OP9 may also be able to use predicted fermentation products of OP9 like hydrogen and some organic acids.

Table 1. Comparisons between Species in the *Thermodesulfobacterium* Genus and other Species of Sulfate Reducers from other Genus-level Groups (from table 1 of Hamilton-Brehm et al., 2013)

Characteristic	OPF15^T	1	2	3	4	5	6	7
Domain	B	B	B	B	B	B	B	A
Morphology	Rod	Oval rod	Rod	Curved rod	Rod	Rod	Rod	Cocci
Dimensions (μm)	0.7 × 2.0	0.5 × 1.0	0.5 × 1.2	0.3 × 1.5	0.3 × 0.9	0.5 × 0.8	0.5 × 2.8	0.5
Motility	−	+	+	+	−	+	−	+
Doubling time (min)	300	240	900	1446	240	186	324	240
Temp. range (°C)	65–90	52–82	65–100	40–70	60–80	50–80	55–74	60–95
Optimal temp. (°C)	83	75	85	65	70	75	70–74	83
G + C (mol%)	30.5	35.2	ND	29.5	34	28	40.0	48.6
Electron acceptors								
Sulfate	+	−	−	+	+	+	+	+
Sulfite	−	−	−	+	+	−	+	+
Thiosulfate	+	+	−	+	+	−	+	+
Elemental sulfur	+	+	−	−	−	−	−	−
Nitrate	−	−	−	−	−	−	−	−
Fe(III) oxide	−	−	+	ND	−	−	−	ND
Electron donors[a]								
H_2	+	+	+	+	+	+	−	+
Formate	+	−	−	+	+	+	−	+
Lactate	−	−	−	+	+	−	+	+
Acetate	−	−	−	−	−	−	−	−
Pyruvate	−	−	−	+	+	−	+	+
Benzoate	−	ND	−	−	−	−	−	ND
Ethanol	−	+	−	−	−	−	−	−

Reference species: 1, *Caldimicrobium rimae* DS^T (Miroshnichenko et al. 2009); 2, *Geothermobacterium ferrireducens* (Kashefi et al. 2002); 3, *Thermodesulfovibrio yellowstonii* YP87^T (Henry et al. 1994); 4, *Thermodesulfobacterium commune* YSRA-1^T (Zeikus et al. 1983); 5, *Thermodesulfobacterium hydrogeniphilum* SL6^T (Jeanthon et al. 2002); 6, *Thermodesulfobacterium hveragerdense* JSP^T (Sonne-Hansen and Ahring 1999); 7, *Archaeoglobus fulgidus* DSM 4304 (Stetter 1988; Zellner et al. 1989). Direct comparisons were performed between *T. geofontis*, *T. commune*, *T. hydrogeniphilum*, and *T. hveragerdense* for electron donor and acceptor experiments

Hypothesis

As described above, attempting to cultivate and isolate OP9, it was found that a novel sulfate reducer, Thermodesulfobacterium, remained present with OP9 as a coculture. Attempts to separate the two by dilution-to-extinction were unsuccessful. This leads to the overarching hypothesis for this thesis project, that OP9 requires *Thermodesulfobacterium* in order to grow, and that this dependence may involve a syntrophic interaction. It is possible that *Thermodesulfobacterium* is utilizing fermentation products like hydrogen and organic acids that OP9 is predicted to produce (Dodsworth et al., 2013).

Alternatively, this syntrophic interaction may be driven by trading of metabolites and/or sulfur compounds. These predictions can be seen graphically in Figure 3. To continue studies on OP9 it is important to study this possible interaction it may be having with this novel species of *Thermodesulfobacterium*. The three main goals of my thesis project, described below, are designed to test the model outlined in Figure 3.

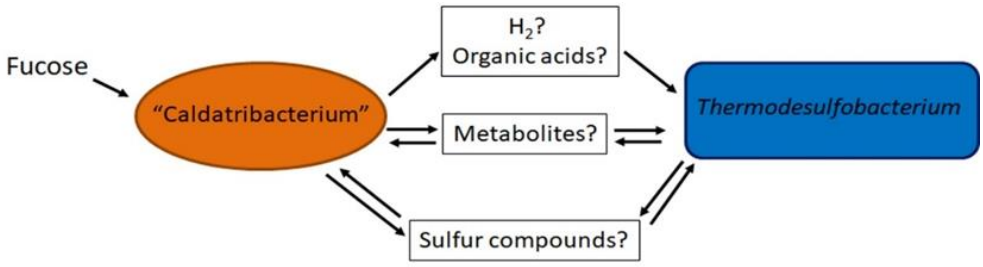

Figure 3. Predictions of Different Substrates being Exchanged between "Caldatribacterium" and *Thermodesulfobacterium*.

Goal 1: Isolation and Characterization of Novel *Thermodesulfobacterium* Species

It is hypothesized that the *Thermodesulfobacterium* can be isolated and grown in pure culture, and that it can utilize hydrogen and organic acids like other sulfate reducers within its genus. Characterization of the isolate may provide insight into which specific products are being exchanged in this interaction (Figure 3). Also, this will provide information needed to formally describe this as a new species and publish this description in the International Journal of Systematic and Evolutionary Microbiology.

Goal 2: Symbiotic Relationship Experiments

To test the nature of the interaction between OP9 and *Thermodesulfobacterium* (Figure 3), various growth experiments will be performed to demonstrate OP9's dependence on the sulfate reducer and probe other possible organisms or substrates that can allow for growth. Dilution-to-extinction is one of the major experiments that will provide insight to the potential symbiotic relationship, specifically by determining whether addition of the isolated *Thermodesulfobacterium* to diluted fucose cultures containing only OP9 cells will allow for growth of these cultures. If the experiments are successful, additional experiments will be performed, like adding supernatant or lysate from the *Thermodesulfobacterium* pure culture or the fucose co-culture, adding different species of *Thermodesulfobacterium* and other sulfate reducers, adding complex organic substrates such as yeast extract, and adding predicted fermentation products that might prevent growth of OP9. These additional experiments can show if OP9 is dependent on a specific partner or substance.

Goal 3: 13C Isotope Labeling Experiments

Stable isotope labeling experiments will be conducted to identify specific substrates that are taken up by the sulfate reducer and OP9. Various 13C labeled substrates will be added to xyloglucan and fucose cultures, and FISH will be performed to identify OP9 and sulfate reducer cells. After FISH, hybridized slides will be sent off to Lawrence Livermore national labs (LLNL) to conduct

nanoscale secondary ion mass spectrometry (NanoSIMS) to determine 13C uptake in individual cells.

CHAPTER TWO

METHODS

Cultivation Conditions for the Co-culture

Base GBS salts medium (Dodsworth et al., 2014) is made with various substrates shown in Table 2. All substrates are added aerobically besides PE salts (a trace element mixture; Dodsworth et al., 2014) and ammonium chloride. The mixture is gassed with N_2 for 1.5 hours before being placed in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA) with a headspace of approximately 90% N_2, 5% H_2, and 5% CO2, where anaerobic conditions are maintained by the presence of a palladium catalyst. Once inside, the PE salts and ammonium chloride are added. The GBS salts media is portioned out to bottles (50 ml into 160 mL bottles, or 10 mL into 25 mL bottles), which are stoppered before being taken out of the anaerobic chamber. Once out of the anaerobic chamber, the bottle headspaces are filled with N_2 to 12 psi and vacuumed for 30 seconds three times, with needles connected to a gassing manifold, and finally filled to 12 psi with N_2. The bottles are then autoclaved and afterwards are stored at room temperature until ready for inoculation. In experiments to test for growth of the co-culture in the absence of sulfate, the sodium sulfate was excluded, the $MgSO4{\cdot}7H_2O$ in the Mg/Ca mix was replaced with $MgCl_2{\cdot}6H_2O$, and sulfate salts of the iron and zinc in the PE salts were replaced with their corresponding chloride salts.

Table 2. Final Concentration of Substrates used to make 1L of GBS Salts Minimal Medium.

Deionized water	Sodium chloride	Potassium chloride	Sodium sulfate	Mg/Ca mix	PE Salts	Ammonium chloride
950 ml	51 mM	1.7 mM	2.1 mM	50 ml	5 ml	2 mM

The co-culture is grown in the bottles with GBS media. Anaerobic, sterile, concentrated substrates added to the medium to the final concentrations shown in Table 3 just prior to inoculation (Dodsworth et al., 2014; Balch et al., 1979). The vitamin mixture contains 2 mg/L biotin, 2 mg/L folic acid, 10 mg/L pyridoxine HCl, 5 mg/L thiamine HCl, 5 mg/L riboflavin, 5 mg/L nicotinic acid, 5 mg/L DL-calcium pantothenate, 0.1 mg/L vitamin B12, 5 mg/L p-aminobenzoic acid and 5 mg/L lipoic acid. Each substrate is added with sterile syringes that are flushed with N2 immediately before being used. A small volume (typically 1/1000 volume) of a previously grown coculture is transferred to the new bottle with substrates and incubated at 73 °C for 7 to 8 days. The culture was examined every other day to ensure growth was occurring. To test whether certain predicted fermentation products produced by OP9 might be inhibitory, sodium lactate, sodium acetate, or sodium formate were added to a final concentration of 1 mM, or the headspace of the medium bottle was pressurized to 10 psi with H_2.

Table 3. Substrates Added to GBS Salts Medium for Routine Growth of the Co-culture

GBS salts medium	**Sodium phosphate (pH 7)**	**Vitamin mix**	**Sodium sulfide**	**Fucose**
50 ml	5 mM	0.2 mL	1.28 mM	1 mM

Cultivating *Thermodesulfobacterium* species and other sulfate reducers

The medium used for all sulfate reducers was the GBS salts medium (Table 2) with alternative substrates added as listed in Table 4. For the related, previously isolated species of sulfate reducers that were obtained from either the DSMZ culture collection (*T. commune*, *T. hveragerdense*) or a co-culture of a related OP9 obtained from a collaborator and isolated by Dr. Dodsworth (*Thermodesulfovibrio yellowstonii*), sodium lactate was added as well to a final concentration of 1 mM to enhance growth.

Table 4. Substrates used for Growth of Sulfate Reducers.

GBS salts medium	Sodium bicarbonate	Sodium acetate	Sodium Thiosulfate	Vitamin mix	Sodium sulfide	Sodium phosphate (pH 7)
50 ml	1 mM	1 mM	1 mM	0.2 ml	1.28 mM	5 mM

Growth on Solid Media

To isolate the sulfate reducer from the OP9 co-culture, streaking for isolation was performed anaerobically in an anaerobic chamber. The same substrates were used for the liquid medium described above (Table 4), except

that sodium sulfide was excluded because the volatile sulfide could poison and inactivate the palladium catalyst in the anaerobic chamber. The medium was solidified by addition of 1% gelrite (gellan gum) and 0.4% of $MgCl_2 \cdot 6H_2O$. To inoculate, approximately 10 µl of liquid culture was dropped onto the plate (or a previously obtained colony was picked), and streaking for isolation was performed using disposable, sterile 10 µL inoculating loops. The plates were placed in an anaerobic incubation vessel (modified 2 L Bandit pressure pots; C.A. Technologies, Louisville, CO, USA). Just before sealing the container, a petri plate with a small paper towel soaked in 1 ml of 5% sodium sulfide was added to the container to provide sulfide as a reductant and potential sulfur source in the gas phase. The container was sealed and pressurized to 10 psi with hydrogen gas. The container was taken out of the anaerobic chamber and placed into a 73 ˚C incubator for up to two weeks. The plates were checked every 3 to 4 days to observe growth and condition of plates. Growth and isolation of OP9 on solid medium was performed similar to that above, except that the substrates listed in Table 3 were used instead and the medium was additionally supplemented with yeast extract and casamino acids at 0.05% final concentration each.

Identification of Isolates

Single colonies were streaked for isolation and were chosen for inoculation into liquid medium made as described above but without gelrite and MgCl2. They were identified by Sanger sequencing of 16S rRNA genes from the

isolates that were extracted and amplified by using the FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA) and polymerase chain reaction (PCR). Primer sets used for PCR were 9bF (5' GRGTTTGATCCTGGCTCAG) and 1406uR (5' ACGGGCGGTGTGTRCAA) and PCR was performed as described (Costa et al., 2009). Products were run on a 1% agarose gel to confirm correct size (~1.4 kb) and were sequenced with the forward and reverse primers at Retrogen (San Diego, CA). Resulting sequences were assembled manually (taking the reverse complement of the reverse primer read using www.bioinformatics.org/sms/rev_comp.html) and compared to sequences in the Genbank database using BLASTn (Zhang et al., 2000). 16S rRNA gene phylogenies were inferred using PhyML (Guindon et al., 2010) as implemented on the website phylogeny.fr (Dereeper et al., 2008).

Characterization of the *Thermodesulfobacterium* Isolate from the Co-culture

Using 25 ml bottles, cultures were grown in 10 ml of media with multiple substrates and incubated at 74 °C. Growth was assessed by visual inspection for turbidity and by phase contrast microscopy. Cell counts were also performed using the Petroff-Hausser counting chamber (Hausser Scientific, Horsham, PA). Maximum/minimum and optimal growth temperatures were measured by growing the *Thermodesulfobacterium* isolate in 25 ml bottles with 10 ml of media in varying temperatures (50°C, 55°C, 60°C, 65°C, 70°C, 74°C, 80°C, and 85°C) and growth was assessed visually and by microscopy. The pH range was assessed

by growing the isolate with sodium phosphate at different pHs (pH 5.5, 5.75, 6.0, 6.25, 6.5, 6.75, 7.00, 7.25, 7.5, 8.00, 8.25, and 8.5) and growth was measured visually and by microscopy.

For genome sequencing a significant amount of DNA was extracted from strain C1. In order to complete these multiple cultures were grown in large bottles containing 50 ml of GBS and incubated for 2 to 3 days (73.5°C). The cells were harvested by placing the cultures in 50 ml conical tubes and centrifuged for 10 minutes. Most of the supernantant was removed leaving only approxiately 1 ml. This remaining one ml is used to resuspend the pellet and placed into a eppendorf tube. The eppendorf tube is spun down for 5 minutes and the supernant is removed. The tubes are placed in the -80°C freezer until DNA extraction is performed.

For short read sequencing, DNA extractions were completed with the FastDNA spin kit from soil (Costa et al., 2009). Samples in elution buffer were sent to Genewiz (www.genewiz.com) for sequencing on the Illumina MiSeq platform (2x250 bp reads). For long read sequencing, DNA was extracted according to the CTAB protocol from Joint Genome Institute (JGI, jgi.doe.gov/user-programs/pmo-overview/protocols-sample-preparation-information/), with some modifications. Briefly, the pellet was resuspended in TE buffer (10mM Tris & 1 mM EDTA) and lysis is performed by treatment with lysozyme (2 mg/mL) for 20 min at 37 °C, followed by proteinase K (1 mg/mL) and SDS (0.5%) for 30 min at 37 °C, and finally hexadecyl-trimethylammonium

bromide (CTAB, 0.9%) at 65 °C for 10 min. These lysates then went through one round of phenol:chloroform:isoamyl alcohol (25:24:1) extraction and one round of chloroform:isoamyl alcohol extraction (24:1), followed by isopropanol precipitation for 2 hours at 4 C. Nucleic acids were pelleted by centrifugation, washed 2x with 70% ethanol, dried, and resuspended in 1xTE with 0.2 mg/mL RNaseA. After incubation for 1 hour at 37 °C, ethanol precipitation was performed. Resulting pellets were resuspended in 50 µL of nuclease free water and quantified by using a Nanodrop spectrophotometer. DNA was sheared by passage through a 26-gauge needle 10 times, and 4 µg of DNA was subsequently used as input for library preparation and sequencing on the Oxford MinION FLO-MIN106 flowcell according to the protocol for 1D Genomic DNA by ligation with kit SQK-LSK108. Long reads and short reads were assembled using Unicycler (Wick et al., 2017). Whole genome in silico DNA-DNA hybridization comparisons were made using the "Genome-to-Genome Distance Calculator" on the website GGDC-DSMZ.de (Meier-Kolthoff et al., 2013).

Dilution-to-Extinction Experiments

To obtain a pure culture of microbes that do not grow on solid medium, one of the techniques used in microbiology is dilution-to-extinction, which was initially done to obtain the co-culture used in this project. As implemented here, dilutions were made from a stock 50 ml sample to 10^{-3} to 10^{-5}-10^{-9} in medium used for the co-culture (Table 3). From a freshly grown co-culture used as inoculum, 0.05 ml was transferred to a new 50 ml bottle to create the 10^{-3}

dilution. From this dilution 0.1 ml was transferred to a new bottle with 10 ml of media to create the 10^{-5} dilution. The 10^{-6} -10^{-9} were then made by transferring 1 mL of the previous dilution to 9 mL of medium. Samples were placed in the 73 °C incubator for 2 to 3 weeks. Samples were observed visually and by microscopy every 3-4 days. Cell counts in cultures used as inoculum were determined using direct microscopic counting with a Petroff-Hausser counting chamber.

Variations on the dilution-to-extinction were used in several different experiments to test for dependence of OP9 on the *Thermodesulfobacterium* isolated from the co-culture (provisionally named *T. auxiliatoris*), and whether other organisms or substrates might also support growth of OP9. In these cases, in addition to cell counts, subsamples of the co-culture used for inoculum were also fixed for FISH (see below). In the first of these, the dilution-to-extinction was performed in replicate as described above. A subset of the replicates were additionally inoculated with ~20 cells/bottle of the *Thermodesulfobacterium* isolate, obtained from a 2-3 day culture of the isolate after cell counting and appropriate dilution in liquid medium. In another version of this experiment, the dilution-to-extinction was performed on the co-culture, and growth was monitored over time. The least-diluted culture that did not exhibit visible turbidity (and based on cell counts and FISH, likely contained just OP9 and not *T. auxiliatoris*) was then used to inoculate a variety of media in quintuplicate, with one quintuplicate additionally receiving 1 drop (~105 cells) of *T. auxiliatoris*. These cultures were incubated, and growth was scored by visible turbidity and microscopy.

Subsequently, this experiment was repeated but additional quintuplicate bottles with additions to test for the ability of other sulfate reducers or other substrates to support growth as shown in Figure 4. To extract supernatant from the co-culture or the *T. auxiliatoris* pure culture, 6 mL of a grown culture was aliquoted into 1.5 mL microcentrifuge tubes and centrifuged for 10 minutes at 13K rpm in the anaerobic chamber. After centrifugation, the supernatant was filtered through two 0.2 µm filters stacked on top of one another. The filtered liquid was placed in a sterile 10 mL bottle to keep anaerobic and stored at 4 °C. The bottle was taken out of the chamber and placed in the fridge for storage. For lysate, the pellets obtained from the lysate production above were resuspended and pooled in 1 mL of medium and sonicated for 15 seconds using a Virtis Virsonic 100 sonicator in the anaerobic chamber. Lysis was confirmed by visual inspection and microscopy. This lysate was then centrifuged, the supernatant was passed through a 0.2 µm filter, sealed in a 10 mL bottle, and stored at 4 °C. To identify whether *T. auxiliatoris* was still present in cultures that exhibited growth (e.g. the cultures to which *T. auxiliatoris* supernatant or yeast extract were added), cells from these cultures were pelleted, DNA was extracted with the FastDNA Spin Kit, and PCR was performed with primers specific for either *T. auxiliatoris* (TDS_1052F, 5’ TCTCTACGCGCTCTAGCACA; TDS_1321R, 5’ GGAGGGCTTTCTGGGATTAG) or OP9 (OP9_16S_F, 5’ AGGAAAGCTGGCCTCTGC and OP9_16S_R, 5’ ACCGTCACAGGAAGGAGC). Thermal cycling parameters used were as follows: 95 °C for 3 minutes; 35 cycles

of 95 °C for 30 seconds, 61 °C or 65 °C for 30 seconds (for *T. auxiliatoris* or OP9 primers, respectively), and 72 °C for 60 seconds; final extension at 72 °C for 6 minutes. Plasmids containing the near-complete 16S rRNA genes from OP9 (pSSW_L1_H02; Costa et al., 2009) or *T. auxiliatoris* (constructed by Rayan Elhamra in the Dodsworth lab using techniques described in Costa et al. (2009)) were used as positive controls at different concentrations.

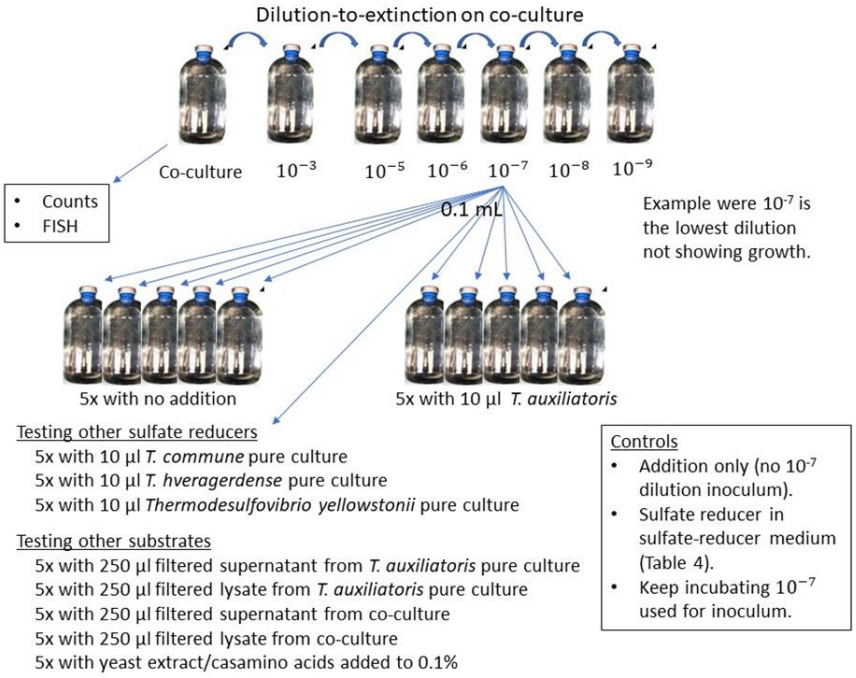

Figure 4. Outline of Dilution-to-Extinction Experiments to Test whether Sulfate Reducers or other Substrates would Support Growth of OP9.

Fluorescence *in situ* Hybridization (FISH)

FISH was performed essentially as described (www.arb-silva.de/fish-probes/fish-protocols/; Fuchs et al., 2007) using the OP9-specific probe OP9-480

(5’ AGCTRTTCACCCCTYCCCTC) labeled with CY3 and the Bacteria-specific probe Bact927 (5’ ACCGSTTGTGCGGGCCC; Simon et al., 2000) labeled with 6-FAM. Prior to FISH, cells from culture samples were pelleted by centrifugation (10k x g for 5 minutes), washed 1x in phosphate-buffered saline (1xPBS), and resuspended in an ice-cold solution of 1% paraformaldehyde as a fixative. Cells were fixed on ice for 1 hour. After fixation, the cells were pelleted, washed three times in 1xPBS, resuspended in 50% ethanol and stored in the -20 ˚C freezer.

Depending on the concentration of cells in the sample, 1-5 µl of the fixed sample was placed onto the wells on gelatin-coated, 10-well glass slides and allowed to air dry. Hybridization solution was made at 30% formamide concentration (Table 5), which was previously determined my me during my undergraduate research project to be optimal for the OP9-specific probe OP9-480 using the Clone-FISH technique (Schramm et al., 2002) and compatible with the Bact-927 probe.

Table 5. Reagents used to make 1.8 mL of Hybridization Solution at 30% Formamide.

4.5 M NaCl	1 M Tris pH 8	Formamide	DEPC treated water	10% SDS
360 µL	36 µL	540 µl	862.2 µl	1.8 µL

Once the hybridization solution is mixed, 10 µl per well in the slide to be hybridized is transferred into a 1.5 ml amber centrifuge tube. For each sample,

0.25 µl of 200 ng/µl probe stock was added to the hybridization solution. A humidity-control chamber was made for each slide using a 50 ml centrifuge tube and a folded kimwipe inserted in the tube (folded 4x, cut an inch off length wise), with the remaining hybridization solution (~1.5 mL) added to soak the kimwipe. Once the chamber is complete, 10 µl of probe/hybridization mixture (yielding 50 ng probe per well) was added to each well containing a sample. The slide was then placed in the humidity-control chamber and incubated at 46˚C overnight for hybridization.

Before taking out the slide from the hybridization chamber, a 50 ml wash solution was prepared for each slide (Table 6) and placed in a 48 ˚C water bath to pre-warm for 20 minutes. Subsequently, the slide is removed from the humidity-control chamber, dipped into DEPC treated water for three seconds, placed into the wash solution and incubated at 48 ˚C for 20 minutes. After the wash, the slides were removed and dipped into DEPC water for three seconds and placed in the fume hood to dry.

Table 6. Ingredients for the FISH Wash Solution.

Formamide	4.5 M NaCl	0.5 M EDTA	1 M Tris pH 8	10% SDS	DEPC water
30%	112.5 mM	5 mM	20 mM	.3 mM	47.20 ml

Once dry, the slides were stained with DAPI (a DNA stain) by immersion in a 1 µg/ml DAPI solution for 3-5 seconds. Immediately after the DAPI stain, the slides

were dipped into DEPC water for 5-10 seconds and air dried in a fume hood. Once dry, 3 µl of a 1:4 mixture of Vectashield: Citifuor was added to each well and a coverslip was placed over the wells. Cells were visualized by epifluorescence microscopy using an Eclipse Ti-U inverted microscope (Nikon, Melville, NY, USA) equipped for epifluorescence with Nikon filter sets compatible with Cy3 (96312 G-2E/C), 6FAM (96343 EN GFP), and DAPI (96310 UV-2E/C), with image capture using a Retiga-SRV camera (QImaging, Surry, BC, Canada) and Nikon Elements v4.13 software.

Fluorescence *in situ* Hybridization (FISH)-Nanoscale Secondary Ion Mass Spectrometry (NanoSIMS)

Another method of examining the relationship between OP9 and *Thermodesulfobacterium* is FISH-nanoSIMS experiments (Carpenter et al., 2013; Dekas et al., 2014), where uptake of 13C by specific cells after incubation with 13C-labeled substrates is assessed using nanoSIMS. Either the OP9/*Thermodesulfobacterium* co-culture grown on ribose or the xyloglucan culture were used in these experiments. Incubations were performed at 73 °C in 25 mL bottles with 10 mL of media prepared as in Table 3, except that fucose was replaced with either 1 mM ribose or 0.02% xyloglucan. These media were inoculated with either the co-culture previously grown on ribose for three transfers or with the xyloglucan enrichment culture that is maintained in the Dodsworth lab. Ribose was used instead of fucose because the co-culture was found to be capable of growth on this substrate, and 13C-labeled ribose was

commercially available for a much lower price than labeled fucose. Once all the bottles reached mid-exponential growth phase (2 days incubation for xyloglucan cultures, 3 days for ribose cultures), 13C-labeled substrates (obtained from Cambridge Isotope Laboratories, Tewksbury, MA) were added to each bottle (Table 7). The samples were placed back in the 73 °C incubator for 2 hours. Once the two hours are complete, 5 mL of sample were harvested and fixed in 1% paraformaldehyde for FISH as described above. FISH was performed on each sample as described aboveon special Arraylt slides, using 4 µl from each sample. Once FISH was complete and hybridization was verified by epifluorescence microscopy, the slides were sent off to Lawrence Livermore National Labs (LLNL) for imaging by nanoSIMS. Images were taken based on using fluorescence microscopy to identify probe hybridization and nanoSIMS to read the ratio of 13C uptake of various substrates (Woebken et al., 2015). FISH and nanoSIMS images were sent back to CSUSB for analysis. The program L'image was used to identify the ratio of 13C uptake of individual cells.

Table 7. Substrates and Final Concentrations used in 13C-labeling Incubations for NanoSIMS Analysis.

^{13}C Carbon-labeled substrates JD529 ribose samples	**^{13}C-Carbon labeled substrates Xyloglucan cultures**
^{13}C sodium bicarbonate (1 mM)	^{13}C algal amino acids (0.01%)
^{13}C sodium acetate (1 mM)	^{13}C algal starch (0.01%)
^{13}C sodium formate (1 mM)	^{13}C sodium acetate (1 mM)
^{13}C ribose (1 mM)	^{13}C sodium bicarbonate (1 mM)
^{13}C ribose (0.0 1%)	^{13}C sodium formate (1 mM)
^{13}C ribose (0.01%) + unlabeled sodium acetate (1 mM)	^{13}C ribose (1 mM)
^{13}C ribose (0.01%) + unlabeled sodium formate (1 mM)	^{13}C glucose (1 mM)
^{13}C ribose (0.01%) + unlabeled sodium bicarbonate (1 mM)	^{13}C xylose (1 mM)
Non-labeled sample	
Control: *Thermodesulfobacterium* isolate in ^{13}C ribose (0.01%)	

Growth Definitions

The tables in the results section will have different indicators of growth. A minus sign (-) will indicate no visible turbity and growth (or under 1x10^6 cells/mL), slight visible growth (or between 1-5x10^6 cells/mL) will be indicated by one plus (+), and clear turbidity will be growth (or above 5x10^6 cells/mL) is two pluses (++).

CHAPTER THREE

RESULTS

Isolation of *Thermodesulfobacterium* from the Fucose Co-culture

Because the non-OP9 member of the co-culture was suspected to be a member of the genus Thermodesulfobacterium, other members of which are known to be sulfate reducers, isolation was attempted by streaking onto solid medium under anaerobic, sulfate-reducing conditions with H_2 as a potential electron donor. Streaking for isolation began using the fucose co-culture, which is the original culture containing OP9 and Thermodesulfobacterium. After approximately one week of incubation, there were multiple small and clear colonies that grew along the streak, as well as some white colonies of the same size and a white precipitate that extended away from the colonies into the medium where colony density was greatest (first part of the streak; Figure 4). Several clear and white, single colonies were individually picked and restreaked 3 times for isolation. Each time, regardless of whether the initial colony used to inoculate the next streak was clear or white, the white precipitate was visible in the part of the streak where growth was most dense, and the most isolate colonies were clear in color. After several rounds of streaking for isolation, two colonies from separate streaks were picked and inoculated into liquid sulfate-reducer medium. Visibly turbid growth was observed in 3-4 days at 73 °C.

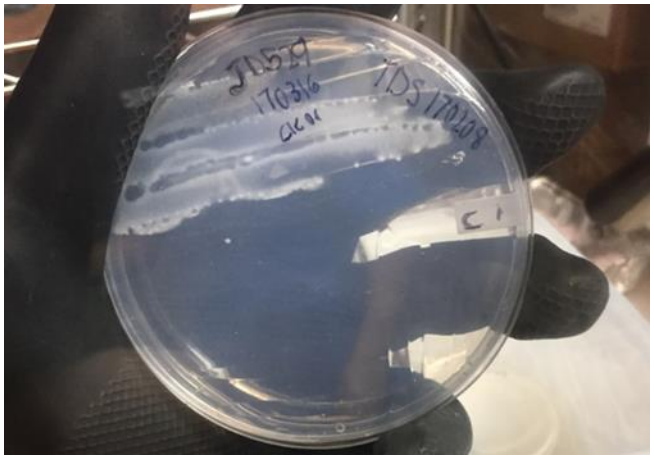

Figure 5. Co-culture Streak for Isolation on Sulfate-Reducer Medium.

A subsample of the liquid culture was used for DNA extraction, 16S rRNA gene PCR, and Sanger sequencing to identify the isolates. BLASTn of the near-full length 16S rRNA gene of the strains had highest hits (94.5-98.5% identity) to members fo the genus *Thermodesulfobacterium*, with the highest identity to *T. hveragerdense* (98.5%), consistent with the isolates being a distinct species within this genus. To further compare the 16S rRNA gene sequences, a phylogenetic tree based on these sequences comparisons was constructed (Figure 6), which offers further support that the isolates are members of the genus *Thermodesulfobacterium*. This tree included the two isolates from the fucose co-culture as well as two isolates obtained using the same techniques described here but with the xyloglucan culture as inoculum (obtained by a previous student in the lab, Joseph Mansuri). All the fucose and xyloglucan culture isolates had identical 16S rRNA gene sequences. Because of this, one of

the fucose isolates was chosen for whole genome sequencing and further characterization. This isolate was named C1 (for “clear colony 1”).

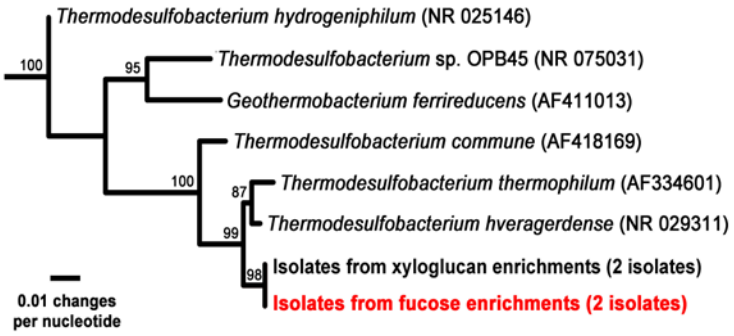

Figure 6. 16S rRNA Gene Phylogeny of the Two Strains Isolated from the Fucose Enrichments, Two Strains Previously Isolated by another student using the same Techniques from the Xyloglucan Enrichments and Described Members of the Genus *Thermodesulfobacterium* and Close Relatives. The Phylogeny was Inferred using PhyML (Guindon et al., 2010) as Implemented in Phylogeny.fr (Dereeper et al., 2008). Numbers at Nodes Represent Bootstrap Support for a given Node (out of 100 bootstrap replicates).

The complete genome sequence of the isolate was obtained by hybrid assembly of short-read and long-read sequence data. The genome consisted of a single, circular 1,829,890 bp chromosome. The in-silico DNA-DNA hybrization (DDH) technique (http://ggdc.dsmz.de; Meier-Kolthoff et al., 2014) was used to measure the degree of relatedness between the C1 isolate and other members of the genus for which genome sequences are available. DDH of the C1 isolate in comparison to all other species yielded values well below the species threshold of 70% (Table 8), providing further evidence that the C1 isolate is a distinct species within this genus. The name *T. auxiliatoris* will be proposed for

this new species, and it will be referred to from now on as *T. auxiliatoris* (or T. aux).

Table 8. Whole Genome comparison of *T. auxiliatoris* (Strain C1) and Related Species by in silico DNA-DNA Hybridization.

	Strain C1	*T. commune*	*T. geofontis*	*T. hveragerdense*	*T. hydrogeniphyilum*	*T. thermophilum*
Strain C1	100%	22.6%	19%	22.5%	17.6%	22.5%
T. commune	22.6%	100%	17.2%	53.1%	17.6%	63.7%
T. geofontis	19%	17.2%	100%	16.4%	18.7%	18.1%
T. hveragerdense	22.5%	53.1%	16.4%	100%	17.3%	56.1%
T. hydrogeniphyilum	17.6%	17.6%	18.7%	17.3%	100%	17%
T. thermophilum	22.5%	63.7%	18.1%	56.1%	17%	100%

Growth Characteristics of the *Thermodesulfobacterium* Isolate

Growth characterisitics of the *Thermodesulfobacterium* isolate were determined by testing for growth with different substrate and under different conditions as shown in Table 9. The only electron acceptor that supported growth was sulfate, and sole electron donors includes hydrogen, formate, and lactate. This makes strain C1 phenotypically distinct from other members of this genus. The use of hydrogen and organic acids supports the hypothesis that it may be comsuming these substrates, putative fermentation products of OP9, in the co-culture. Growth temperature range was seen from 50-80˚C with optimal temperature at 74˚C, and the pH range was determined to be from 5.5 to 8.0 with an optimal pH at 7.0, broadly similar to other members of this genus.

Table 9. Characteristics of Strain and the other Species in the *Thermodesulfobacterium* Genus.

	C1 Isolate	*T. geofontis*	*T. commune*	*T. hydrogeniphilum*	*T. hveragerdense*
Electron acceptors**					
2 mM sulfate	+	+	+	+	+
2 mM sulfite	-	-	+	-	+
2 mM thiosulfate	-	+	+	-	+
Elemental sulfur	-	+	-	-	-
Electron donors**					
10 psi H_2	+	+	+	+	-
1 mM formate	+	+	+	+	-
1 mM lactate	+	-	+	-	+
1 mM acetate	-	-	-	-	-
1 mM pyruvate	-	-	+	-	+
Benzoate	-	-	-	-	-
Ethanol	-	-	-	-	-
0.025% fucose	-	NR	NR	NR	NR
0.025% glucose	-	NR	NR	NR	NR
0.025% xyloglucan	-	NR	NR	NR	NR
Growth temperature					
Minimum/Maximum	50°C-80°C	65°C-90°C	60°C-80°C	50°C-80°C	55°C-74°C
Optimum Temparture	74°C	83°C	70°C	75°C	70°C-74°C
pH range					
Minimum/Maximum	5.5-8.0	6.0-8.5	6.0-8.0	6.3-6.8	NR
Optimum pH	7.0	6.5-7.0	7.0	6.5	7.0

Dilution-to Extinction/Symbiotic Relationship Experiment

It is hypothesized that OP9 does not grow at the higher dilutions because of the absence of *T. auxiliatoris* at the higher dilutions. *T. auxiliatoris* may be consuming or producing products that allows OP9 to grow. By adding *T. auxiliatoris* in the dilutions it is predicted that OP9 will be able to grow at the higher dilutions. Control groups for this previous experiment was *T. auxiliatoris*

alone in OP9 medium (negative) and *T. auxiliatoris* in its own medium (positive). The cultures were grown for up to 3 weeks, and samples that grew before that time were taken out of the incubator to retain viability of the sample. In all three samples for both sets of triplcates for the first dilution to extinction experiment, only 10^{-5} and 10^{-6} showed visible growth. These results suggested that *T. auxiliatoris* may not hold a syntrophic relationship and improve overall growth of OP9. As expected, the negative control did not grow, and the positive control grew. This indicates *T. auxiliatoris* was viable in the experiment and cannot grow alone in OP9 medium. Table 10 summarizes the method and result of the dilution to extinction experiment described above.

The original GBS media contained sulfate that could be used by *T. auxiliatoris* in the co-culture. During initial attempts to separate OP9 and *T. auxiliatoris* the sulfate was removed when creating the GBS media. However, even with the removal of sulfate, *T. auxiliatoris* was still present with OP9. This was demonstrated by isolation of *T. auxiliatoris* on solid media from fucose cultures grown in the sulfate-free media. When the media was used to grow cultures, the growth of the culture would take 10 days rather than the normal 6 day period. It is assumed that this is happening due to lower levels of T. aux present. Whem FISH was conducted on the cultures a signficantly less amount of T. aux was present which strengthened this assumption. This experiment was completed again with normal GBS media containing sulfate and simialr results

were obtained, with growth occurring or not occurring at the same dilutions with or without added *T. auxiliatoris* at inoculation.

Table 10. Dilution-to-Extinction Experiment completed in Triplicate. A Triplicate set without *T. auxiliatoris* (T. aux) and with *T. auxiliatoris*. Cell Count Indicates the Predicted Number of Total Cells in the Dilutions based on Direct Cell Counts on the Culture used for Inoculum.

Sulfate-free GBS medium	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
W/O T. aux 1	++	+	-	-	-
W/O T. aux 2	++	-	-	-	-
+W/O T. aux 3	++	+	-	-	-
_____	_____	_____	_____	_____	_____
With T. aux 1	++	++	-	-	-
With T. aux 2	++	++	-	-	-
With T. aux 3	++	++	-	-	-
Cell count	725 cells/ml (7250 cells per bottle)	72.5 cells/ml (725 cells per bottle)	~7.3 cells/ml (73 cells per botte)	Approx. one cell per/ml (7.3 cells per bottle)	One or no cells

When inspected by phase contrast microscopy, it was seen that a small abundance of cells was in the higher dilutions (~10^5 cells/ml), e.g. in the 10^{-7} dilutions, but these dilutions never became turbid like the lower dilutions. This suggests that OP9, on its own, is able to grow to very low densities but is inhibited from further growth, possibly because of buildup of waste products or a

lack of important growth factors. To test this, another round of triplicate dilutions to extintion was performed, without added *T. auxiliatoris*. One sample was taken that showed the presence of cells (~105 cells/mL) by microscopy but not visible turbidity (10^{-6} dilution), which potentially contained OP9 but no

Thermodesulfobacterium. This 10^{-6} dilution was used to inoculate 6 bottles of sulfate-free GBS fucose medium, 0.1 ml per bottle. However, three out of the six bottles were additionally inoculated with one drop (10 µL) of *T. auxiliatoris* pure culture. After one week of incubation, two of the three bottles with *T. auxiliatoris* grew to turbidity, while the other bottles did not exhibit visible growth after two weeks incubation (Table 11). As a negative control, three bottles GBS fucose media were inoculated with one drop of *T. auxiliatoris*, but no inoculum from the 10^{-6} dilution, to ensure that the *T. auxiliatoris* did not show growth on its own. As an additional negative control, the 10^{-6} dilution used for inoculum was returned to the incubator. Dilutons were kept in the incubator for up to 3 to 4 weeks. Any dilutions that grew optimally were removed for sampling and to keep viability. As positive control for growth of *T. auxiliatoris*, GBS sulfate reducer medium was inoculated with the *T. auxiliatoris* pure culture. As expected, the negative controls did not grow, but the positive control grew. This confirmed that *T. auxiliatoris* was viable and but not capable of growth on fucose, as expected. The results for this experiment are summed up in Table 11. The results showed that adding *T. auxiliatoris* to OP9 in higher dilutions that did not show turbidity, but showed growth by microscopy, was able to support growth of OP9.

Table 11. Follow up Experiment after Dilution-to-Extinction to Observe Growth with or without *T. auxiliatoris* (T. aux)

Dilution 10^{-6}	**1**	**2**	**3**
W/O T. aux	-	-	-
With T. aux	++	++	-

This experiment was repeated, but with two sets of quintuplicate cultures inoculated with a similar dilution-to-extinction of the co-culture, using the lowest dilution that did not exhibit visible growth as inoculum. It was found that all five of the samples with both OP9 and *T. auxiliatoris* grew, but none of the five culturers with just OP9 (to which *T. auxiliatoris* was not added) did not grow (Table 12). Unfortunately, the samples used for inoculum were not fixed and measurements to idenitfy ratio of both bacterium by microscopy could not be performed. This experiment needs to be repeated with samples being fixed for FISH experiments. Nonetheless, these results suggsted that *T. auxiliatoris* was sufficient to support growth of OP9.

Table 12. Results for Quintuplicate OP9 Experiment with no Control

	1	2	3	4	5
OP9 Alone	-	-	-	-	-
OP9 + T. aux	++	++	++	++	++

Testing whether other Sulfate Reducers can Support Growth of OP9

To follow up on the previous experiment, different sulfate reducers were tested to see if they too could support growth of OP9, or whether this ability was specific to *T. auxiliatoris*. Because these other sulfate reducers can utilize different substates as electron donors in comparison to *T. auxiliatoris* (Table 1), it is possible that OP9 may or may not be able to support their growth depending on the fermentation products that it produces. The experimental setup is shown in Figure 4 ("testing other sulfate reducers"), using a 10^{-6} diluiton in a dilution-to-extinction series of the co-culture. In this experiment, none of the cultures that presumably contained OP9 alone (no addition of sulfate reducer) showed visible growth, but all five replicates to which *T. auxiliatoris* or the three other sulfate reducers were added grew to visible turbidity (Table 13).

Table 13. Ability of Various Sulfate Reducers to Support Growth of a Diluted (10^{-6}) Co-culture Sample. Abbreviations: *T. commune* (*T. com*), *T. hveragerdense* (*T. hver*), *Thermodesulfovibrio yellowstonii* (*T.vib*), *T. auxiliatoris* (*T.aux*)

Repliate number	10^{-6} only	10^{-6} + *T.aux*	10^{-6} + *T.com*	10^{-6} + *T.hver*	10^{-6} + *T.Vib*
1	-	++	++	++	++
2	-	++	++	++	++
3	-	++	++	++	++
4	-	++	++	++	++
5	-	++	++	++	++

Testing whether different Substrates can Support Growth of OP9

As explained perviously dilution-to-extinction was performed in order to reterive the diluted culture of OP9. The starting cell count for the sample used in the experiment was $4.5x10^7$ cells/mL. When dilution-to-extinction was performed it was found that the 10^{-7} did not grow to turbidity, but a few cells were observed by phase contrast microscopy. This 10^{-7} dilution was used to inoculate media with various additions as shown in Figure 4 (“testing other substrates”), along with accompanying controls.

The results of the experiment are shown in Table 14. For cultures inoculated with the 10^{-7} dilution only, 3 out of the 5 eventually showed some turbidity after prolonged incubation. However, when these samples were inoculated into fresh media, growth was not seen visually or by microscopy. All five replicates to which *T. auxiliatoris* was added showed growth after 7 days, as expected based on previous experiments. Interestingly, all five replicates to which yeast extract a casamino acids, or supernatant from the *T. auxiliatoris* pure culture, were added showed growth after four and seven days, respectively. This suggested that *T. auxiliatoris* produces some soluble substrate that can support growth of OP9 and, importantly, these cultures potentially represented pure cultures of OP9. In contrast, only one of the replicates to which *T. auxiliatoris* lysate was added showed growth after 14 days, and no growth was observed in

any of the replicates to which co-culture supernatant or lyaste were added showed growth. The results for the controls were all negative as seen in table 14. For *T. auxiliatoris* the positive and negative controls are not seen in table 14. However, the controls indicated that *T. auxiliatoris* was viable as it grew in its own media but were not viable with fucose as a sole carbon source, as expected.

Table 14: Ability of Various added Substrates to Support Growth of a Diluted (10^{-7}) Co-culture sample. Abbreviations: *T. auxiliatoris* (T. aux) and Yeast Extract and Casamino Acids (YE/C).

Replicate number	10^{-7} only	10^{-7} + T. aux	10^{-7} + YE/C	10^{-7} + T. aux supernatant	10^{-7} + T. aux lysate	10^{-7} + co-culture supernatant	10^{-7} + co-culture lysate
1	±*	++	++	++	-	-	-
2	±*	++	++	++	-	-	-
3	±*	++	++	++	-	-	-
4	-	++	++	++	-	-	-
5	-	++	++	++	++	-	-
Uninoculated Control (no 10^{-7})	-	-	-	-	-	-	-

*, Some growth was observed after prolonged incubation (>3 weeks), but when these cultures were used to inoculate fresh media, no growth was observed.

PCR with primers specific for *T. auxiliatoris* on DNA extracts from all five replicates to which *T. auxiliatoris* cells, *T. auxiliatoris* lysate, and yeast extract/casamino acids were added confirmed that *T. auxiliatoris* was detectable

in the cultures where this strain was added, but was not detected in the cultures that exhibited growth when *T. auxiliatoris* supernatant or yeast extract were added (Figure 7).

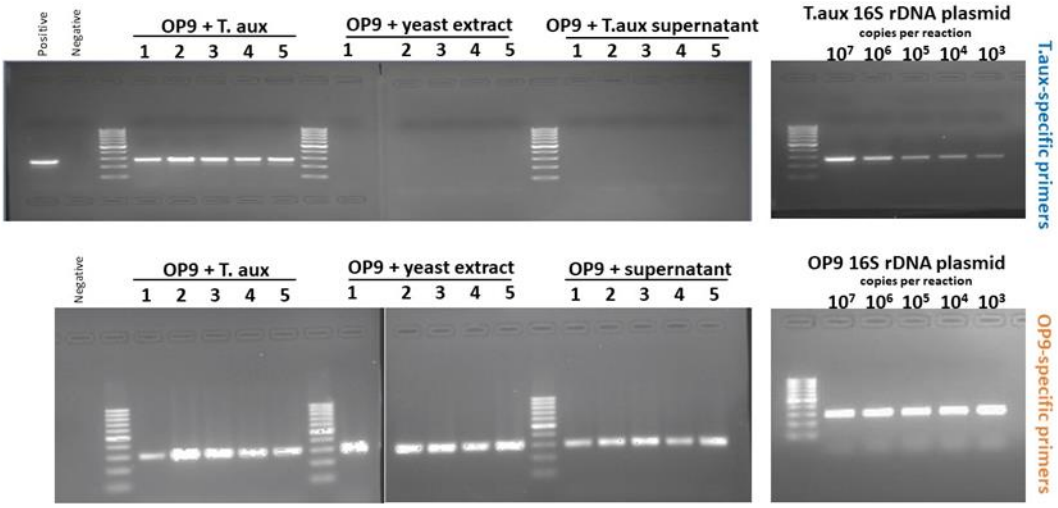

Figure 7. PCR Screen for the Presence of *T. auxiliatoris* in Replicate Cultures that Exhibited Growth in Table 16. For each set of Five Replicates, Labels and Numbers Correspond to Experiments and Replicate Numbers in Table 14. The Figure is a Composite Image of Several Ethidium Bromide-stained 2% agarose gels viewed using a UV Transilluminator. Product with Primers Specific for *T. auxiliatoris* (T. aux) Indicates its Presence (detectable to 103 16S rRNA gene copies per reaction), while Product with Primers Specific for OP9 Confirm the Presence of Amplifiable DNA in the Extracts.

DNA extracts from replicates 1 and 5 to which yeast extract/casamino acids were added were uses as template in PCR with bacterial specific primer set 9bF/1406uR, and products were Sanger sequenced with the 9bF primer. Both sequences obtained were identical, and BLASTn the sequences showed 99.75% identity to the 16S rRNA gene clone SSW_L1_H02, obtained from a

spring nearby GBS (Costa et al., 2009), and showed 100% identity to the 16S rRNA gene sequence from the OP9 metagenome bin from the fucose co-culture (Figure 2).

Isolation of OP9

Due to OP9 potentially being isolated, another test was performed to identify what substrate allowed for the growth of OP9. As seen in Table 15, it was found that yeast extract was the substrate that allowed for the growth of OP9, but only in the presence of fucose. Casamino acids did not support growth, and neither did the substrates acetate, thiosulfate, and bicarbonate, trace amounts of which would likely have been present in the *T. auxiliatoris* pure culture lysate used for the experiment in Table 14.

Table 15. Testing OP9 with Different Substrates: Fucose, Yeast Extract (Y), Casamino Acids (C), Acetate (A), Thiosulfate (T), and Bicarbonate (B)

	Fucose	Fucose + Y + C	F + Y	F + C	Y + C	F + A + T + B
OP9 5	-	++	++	-	-	-

To ensure OP9 was isolated, streak plating was performed. Previously plating with just the normal substrates used to inoculate OP9 cultures did not allow OP9 to grow plates. However, using the two substrates, colonies formed on the plates (Figure 8). With inoculation of OP9 it was confirmed that OP9 had

been isolated. DNA extractions were performed, and samples were sent of for sequencing to confirm the results. With the results confirming pure OP9 it was official that OP9 had been isolated.

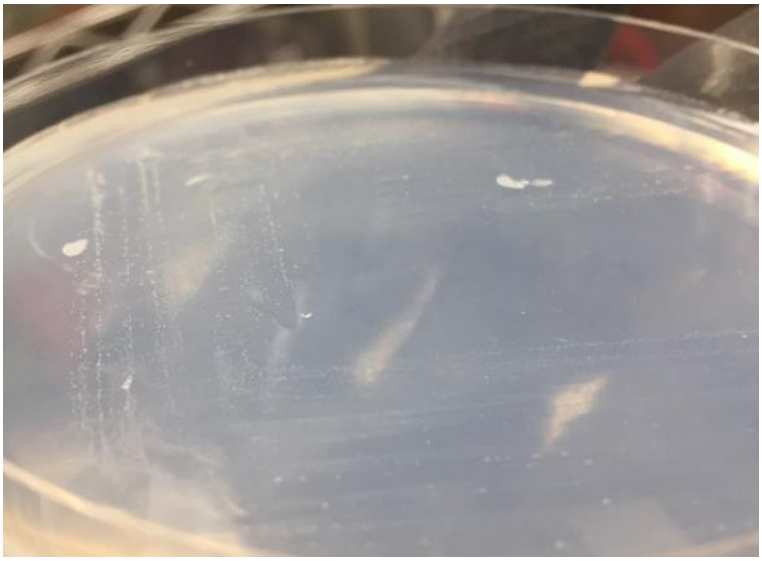

Figure 8. Image of Streak for Isolation of OP9

Testing percent amount needed of yeast extract to grow OP9

When yeast extract was initially used in experimentation with OP9, the final concentration was 0.05%. In this experiment, percent amount of yeast extract needed by OP9 was tested. OP9 was tested with yeast extract at lower concentrations. Results indicated that 0.001% yeast extract was enough to allow growth of OP9 to a high density, although concentrations as low as 0.0001% yeast extract allowed for some growth (Table 16)

Table 16. Results for OP9s Growth with Different Amounts and Percentages of Yeast Extract.

Dates	0.05%	0.01%	0.005%	0.001%	0.0005%	0.0001%
03/29/19 (inoculation)	-	-	-	-	-	-
04/02/19	+	+	+	+	-	-
04/05/19	++	++	++	++	+	+

Dilution-to-Extinction with Isolated OP9

To verify isolation of OP9, dilution to extinction was completed in triplicate three separate times, but now including yeast extract. The results indicated that yeast extract allowed growth for isolated OP9 for almost all dilutions. In the first and second dilutions, OP9 grew up to 10^{-8} and in the third dilution OP9 grew up to 10^{-9} (Table 17), all of which would be expected to contain only a few OP9 cells, contrasting with results when diluting the co-culture (Table 10) and consistent with this culture representing an isolate of OP9.

Table 17. The Results of Three Different Dilution-to-Extinction Experiments with OP9 and Yeast Extract.

Dilution round 1	10^{-2}	10^{-4}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
# of triplicate cultures with growth	3	3	3	3	3	-	-
Estimated cells/bottle*	$1.5x10^6$	$1.5x10^4$	150	15	~1-2	<1	<<1
Dilution round 2	10^{-2}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
# of triplicate cultures with growth	3	3	3	3	3	3	-
Estimated cells/bottle*	$8x10^6$	$8x10^4$	8000	800	80	8	<1
Dilution round 3	10^{-2}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
# of triplicate cultures with growth	3	3	3	3	3	3	1
Estimated cells/bottle*	$3.1x10^7$	$3.1x10^5$	$3.1x10^4$	3100	310	30	3

***** Cells per Bottle (10 mL of medium) were Estimated Based on Cell Counts in the Cultures used for Inoculum (cells/ml): 1., $1.5 \, x \, 10^7$; 2, $8 \, x \, 10^7$; and 3, $3.1 \, x \, 10^8$.

In Dr. Dodsworth's lab, one other culture named "Southern Illinois University Carbondale" (SIUC) containing a related OP9 (~98% identity to the OP9 in the fucose co-culture) was obtained from a collaborator, Dr. Scott Hamilton-Brehm. The culture was obtained from a borehole (~500 m depth) in southern Nevada. This SIUC culture appeared to be a co-culture composed mostly of OP9 and a close relative (99.8% 16S rRNA gene identity) of *Thermodesulfovibrio yellowstonii*, a sulfate reducer unrelated to *T. auxiliatoris* based on 16S rRNA gene sequencing. This related species had similar issues

with isolation from its co-culture, as repeated dilution-to-extinction did not result in removal of the *Thermodesulfovibrio*. However, due to the results with my OP9, streaking for isolation of this related OP9 (termed “SIUC”) was performed on plates with fucose, yeast extract and casamino acids. The results were similar, with SIUC forming colonies. After three successive streaks for isolation, a colony of SIUC was inoculated into liquid medium with fucose and was able to grow. 16S rRNA gene sequencing on DNA extracts from this culture confirmed the identity of the isolate.

With SIUC and OP9 isolated, a set of tests similar to those in Tables 12 and 13 was performed with these isolates (rather than dilutions from co-culture) using different *Thermodesulfobacterium* species and substrates. The results confirmed the ability of all four sulfate reducers, as well as yeast extract (filter sterilized as usual, or autoclaved) and *T. auxiliatoris*, to support growth of these isolates (Table 18). Additionally, a minimal amount of growth was observed when *T. auxiliatoris* lysate was added.

Table 18. Testing whether different Species of *Thermodesulfobacterium* and Substrates Support Growth of the OP9 and SIUC isolates. Abbreviations: Yeast Extract (YE, 0.05% final concentration), *T. commune* (*T. com*), *T. hveragerdense* (T. hver), *Thermodesulfovibrio yellowstonii* (*T. vib*), *T. auxiliatoris* (*T. aux*), and Autoclaved Yeast Extract (AC_YE, 0.05% final concentration).

	Control (OP9 isolate only)	**Isolate + YE**	**Isolate + T. com**	**Isolate + T. hver**	**Isolate + T. vib**	**Isolate + T. aux**	**Isolate + AC_YE**	**Isolate + T. aux supernatant**	**Isolate + T. aux Lysate**
OP9	-	++	++	++	++	++	++	++	+
SIUC	-	++	++	++	++	++	++	++	+

Testing for Inhibition of Growth of the OP9 Isolate by Potential Fermentation Products

OP9 was tested with its own predicted fermentation products to see if they would inhibit growth of OP9 (Dodsworth et al., 2013). The substrates that were tested and the amount used with OP9 were H_2 (10 psi), 1 mM sodium lactate, 1 mM sodium formate, and 1 mM sodium acetate. The results showed no siginficant difference with cell counts (cells/ml) ranging between 4-8x10^7 which are similar to OP9 grown under normal conditions.

FISH-nanoSIMS Analysis

FISH-nanoSIMS was used to detect uptake of 13C-labeled compounds by OP9 in the xyloglucan enrichment cultures and by the OP9-*T. auxiliatoris* co-culture grown on ribose (instead of fucose). In the xyloglucan culture, OP9 was observed to incorporate 13C when incubated with 13C-labeled ribose, glucose, and xylose (Figure 9). In the case of xylose, uptake by OP9 was considerably

higher in comparison to other cells (those not positive for FISH probe OP9-480). OP9 also incorporated 13C when the culture was incubated with 13C-labeled amino acids, and to a much lesser extent 13C-acetate. This contrasts with results obtained by other lab members that have found that xylose, amino acids, and acetate do not support growth of the co-culture, at least when provided as sole carbon sources.

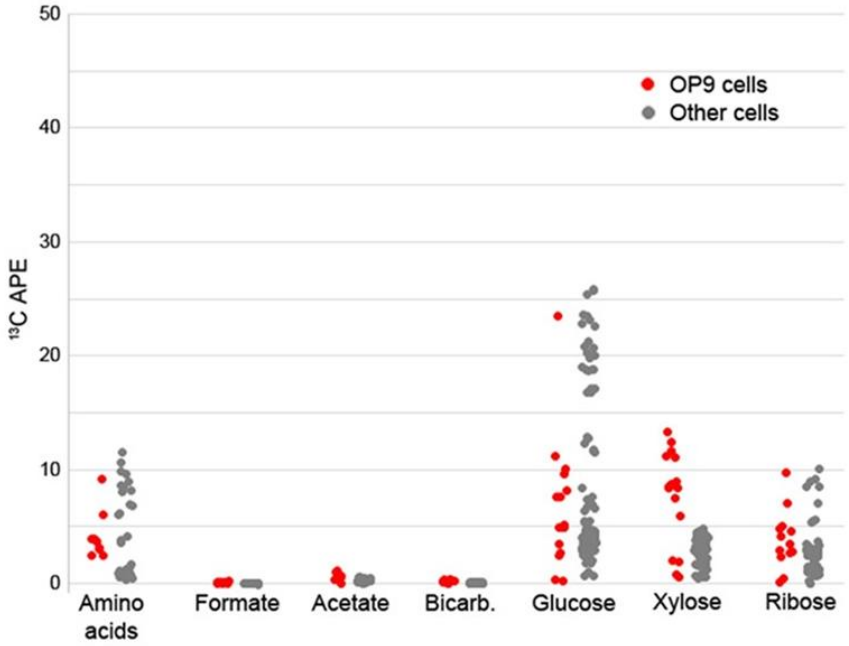

Figure 9. Uptake of 13C in Xyloglucan Cultures Amended with Various Substrates. OP9 cells and “other cells” Represent Cells that were Positive or Negative, Respectively, for FISH using Probe OP9-480; both were Positive for DAPI staining. 13C-labeled Compounds were added to a Final Concentration of 0.01% (amino acids) or 1 mM (other substrates) to 2-day Xyloglucan Cultures and Incubated at 73 °C for 2 hours before Harvest and Fixation. Labeling is expressed as Atom Percent Excess (APE) of 13C (Amount of 13C above Natural Abundance) as Determined by nanoSIMS, with each point Representing a Single cell. Bicarb, Bicarbonate.

In the co-culture grown on ribose, uptake of 13C-labeled ribose or possible fermentation products was assessed, as well as the effect of adding possible (unlabeled) fermentation products on uptake of 13C-ribose. The first time that nanoSIMS was performed, FISH was only done with the OP9-480 probe, and uptake of 13C by all cells (DAPI-positive, regardless of probe signal) was determined using an automated analysis. Subsequently, FISH was re-done on these samples with both the OP9-specific probe and a probe that should bind to most bacteria (including OP9 and *T. auxiliatoris*), which allowed for manual differentiation of OP9 cells (positive for both probes) and *T. auxiliatoris* cells (positive for the bacterial probe only). It was hypothesized that *T. auxiliatoris* might be taking up inorganic carbon, formate or acetate produced by OP9 growth on 13C-ribose. If this were the case, then 13C-labeling of *T. auxiliatoris* would be expected to some extent in the co-culture incubated with 13C-ribose, but this labeling would decrease when unlabeled substrates subject to cross-feeding (bicarbonate, formate or acetate) were added along with 13C-ribose. However, little 13C was observed in the few *T. auxiliatoris* cells analyzed in the 13C-ribose incubations (Figure 10), even though uptake of 13C-bicarbonate and 13C-acetate were observed by both organisms and labeling of OP9 was clearly observed in the presence of 13C-ribose. Interestingly, labeling of OP9 by 13C-ribose appeared to be stimulated when unlabeled bicarbonate or acetate were added, possibly due to stimulation of the *T. auxiliatoris* by these substrates. The pure culture of *T. auxiliatoris* showed only minimal uptake of 13C-ribose, as

expected. Of the nanoSIMS images obtained, only a few *T. auxiliatoris* cells were present, resulting in a very low sample size, and additional imaging and analysis would be necessary to make firm conclusions.

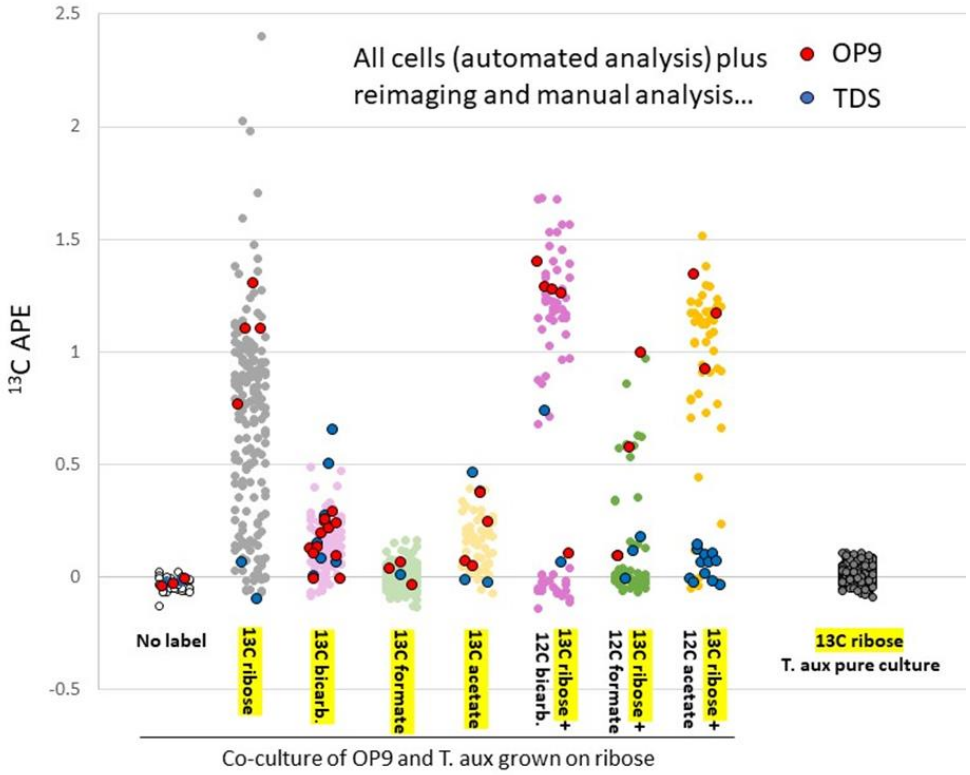

Figure 10. Uptake of 13C by OP9 and *Thermodesulfobacterium* (T. aux) in Co-culture or *Thermodesulfobacterium* in Pure Culture. A subset of Specific Cells were Analyzed Manually: OP9 Cells (red dots with black outlines) were Identified as being Positive for Cy3-labeled OP9-480 FISH probe; *Thermodesulfobacterium* Cells (blue dots with red outlines) were Negative for the OP9-480 Probe but Positive for 6FAM-labeled Bact-927 Probe and DAPI. Other dots Represent individual Cells Identified in an Automated Bulk Analysis, Regardless of Probe Staining. 13C-labeled (yellow highlight) and Unlabeled Compounds were Added to a Final Concentration of 0.01% to 3-day Cultures and Incubated at 73 °C for 2 hours before Harvest and Fixation. Labeling is Expressed as Atom Percent excess (APE) of 13C (amount of 13C above natural abundance) as determined by nanoSIMS.

CHAPTER FOUR

DISCUSSION

Isolation and Characterization of *Thermodesulfobacterium auxiliatoris*

Currently the paper is being finalized and should be completed early August 2019. Also, certificates of deposit from German (DSMZ) and Japanese (JCM) strain collections have been received. A paper describing the isolate as a new species will be submitted to the International Journal of Systematic and Evolutionary Microbiology.

Isolation of Novel “Caldatribacterium”

In the end, I was able to obtain a pure culture of OP9. Three major experiments provide substantial evidence that OP9 is in fact isolated: initial dilution tests with yeast extract, dilution-to-extinction with the putative isolate, and obtaining isolate colonies after plating on solid medium with yeast extract. In the initial isolation in Table 14, multiple substrates were tested with diluted OP9 in order to better understand what allows OP9 to grow. One of tests used both yeast extract and casamino acids as growth substrates. Shortly after incubation OP9 grew and this suggested that OP9 was isolated. Further tests indicated that the yeast extract was the main substrate that allowed the growth of OP9 (Table 15). To ensure OP9 was purified, dilution-to-extinction and streak for isolation was performed. Originally dilution-to-extinction would result in OP9 growing only in the lower dilutions in the absence of yeast extract (Table 10). With yeast

extract, OP9 grew at the higher dilutions, where only one or a few cells were likely present (Table 17). For plating, in the presence of yeast extract along with normal growth substrates (fucose), colonies formed on the plates and were picked for culturing (Figure 8). These experiments provided the confidence to say that OP9 was in fact isolated. However, to provide further evidence of isolation, whole genome sequencing and FISH experiments will be performed in the future. Also, a full phenotypic characterization, similar to that done with *T. auxiliatoris*, can now be completed with OP9.

Nature of the Interaction between OP9 and *T. auxiliatoris*

The fact that it was apparently not possible to separate OP9 from *T. auxiliatoris* in the co-culture in the absence of yeast extract gave the opprtunity to study the interaction between the two microbes. The first objective was to characterize *T. auxiliatoris* to find out what substrates were potentially being traded between OP9 and *T. auxiliatoris*. With previous metagenomic data, it is predicted that OP9 produces substrates like H_2, formate, and acetate, which are substrates *T. auxiliatoris* is known to use as growth substrates (Dodsworth et al., 2013). *T. auxiliatoris* cannot use acetate alone, but it is known that acetate improves the growth of the cultures. For OP9, it was unknown what types of substrates produced by *T. auxiliatoris*, if any, were being taken up or utilized. It was hypothesized that *T. auxiliatoris* allowed for growth of OP9 by consumption of waste products (in a classical syntrophic interaction), or alternatively that vital metabolites or sulfur compounds produced by *T. auxiliatoris* were being

consumed by OP9 (Figure 3). To better understand this complex relationship, further tests were performed.

The first experiment to narrow down what substrate(s) were potentially being traded was from the experiment in Table 14. As previously mentioned, it was found yeast extract allowed OP9 to grow. With this result, it became less likely that sulfur compounds were being traded, at least sulfur compounds that might be specifically produced by sulfate reducers. Currently, it is unknown what component of yeast extract OP9 is using for growth. However, this suggests that whatever *T. auxiliatoris* is producing, it may not be specific to only *T. auxiliatoris*, but rather it is a more common compound(s) being produced. The next substrates tested were *T. auxiliatoris* supernatant and lysate. These substrates were tested in order to assess if *T. auxiliatoris* was making a soluble substrate that OP9 needed or if OP9 was consuming something off *T. auxiliatoris* cells itself. Also, it would answer the question if OP9 needed *T. auxiliatoris* viable and present in order to grow. As seen in Table 14, all 5 of the supernatant cultures grew and only 1 lysate culture grew. This further strengthens the idea that OP9 requires something soluble that *T. auxiliatoris* is producing, and not the cell itself. Although the supernatant was able to be separated from *T. auxiliatoris* cells, it still contained growth substrates of *T. auxiliatoris*. As seen in Table 15, the substrates were tested separately with OP9 and the culture did not grow. The last substrate to be tested was the OP9 co-culture supernatant and lysate. This test was to see if any substrates or cell products were being produced in co-

culture that allowed for growth. However, the results showed no growth in any of the 5 cultures for both the supernatant and lysate. The lack of growth with the OP9 supernatant may be due to *T. auxiliatoris* only being present in the co-culture at 5%. With a small percentage, *T. auxiliatoris* may have not been able to produce enough of the substrate that OP9 needs in order to grow.

As previously hypothesized, OP9 was potentially being inhibited by its own products and needed *T. auxiliatoris* to consume these products. Based on previous metagenomic data (Dodsworth et al., 2013), OP9 is hypothesized to produce various fermentation products like hydrogen and organic acids like acetate. Lactate and formate were also tested because *T. Auxiliatoris* could use these substrates as electron donor. To test this, isolated OP9 was grown with these substrates at a high concentration. It was found that OP9 was still able to grow in the presence of these potential fermentation products, with total growth yields essentially the same as cultures grown without these additions. It is safe to assume *T. auxiliatoris* is consuming the products of OP9, but this result suggests the purpose of *T. auxiliatoris* is more likely a metabolite being produced that OP9 needs.

Specificity of the Interaction

OP9 was tested with different sulfate reducers, *T. commune*, *T. hveragerdense*, and *Thermodesulfovibrio yellowstonii*. This experiment was performed in order to test for specificity of the relationship and to compare characteristics among the sulfate reducers. In the first experiment (Table 13),

diluted cultures of OP9 were used and each sulfate reducer was added individually. Interestingly, all 3 of the related species grew with OP9. Also, in Table 18 the experiment was repeated, but isolated OP9 and SIUC OP9 were used and the same results occur. Interestingly OP9 and SIUC OP9 were able to swap suflate reducers and still grow (SIUC originally containing *T. yellowstonii*). Both results provide evidence that the growth-promoting compound or compounds being produced are not specific to *T. auxiliatoris* but can be produced by a wide range of suflate reducers, and that similar growth-promoting compound(s) are present in other microorganisms like yeast. In the future, non-sulfate reducers can be tested to see if they produce similar outcomes. Currently in lab, two microorganisms (*Dictyoglomus* and *Thermotoga* spp.) that abundant in the xyloglucan culture from which the co-culture was derived, have been isolated. Determining whether these isolates can also support the growth of OP9 in the absence of yeast extract would further help to determine which types of organisms are capable of this.

NanoSIMS Analysis

In the xyloglucan cultures, OP9 was able to incorporate carbon from xylose, ribose and glucose (Figure 9). It was expected to observe uptake of glucose and ribose, because these substrates have been shown to support growth of the co-culture by other members of the lab. However, the co-culture is not able to grow on xylose, even though OP9 appears to specifically take up xylose in the xyloglucan cultures. It is possible that OP9 may play an important

role in xylose uptake in the xyloglucan cultures, but that it is not able to grow on this as a sole carbon source. Although there was some uptake of the amino acids by OP9, growth does not occur solely on this substrate in the co-culture either. It is unclear if amino acids play a role in growth and further testing is required. Potentially testing separate amino acids could be beneficial in finding specific ones that show uptake. Specifically, tryptophan is not in the casamino acids that was used and would be interesting to test. As predicted and tested, acetate, formate, and bicarbonate do not assist in growth of OP9 separately or together.

In general, Figure 10 shows mixed results, with both OP9 and *T. auxiliatoris* cells showing 13C uptake at a range of levels depending on the substrate. This may be due to differences in the level of activity among cells, and this can be seen especially in the 13C ribose. Second, the results suggest that sodium bicarbonate and acetate may be stimulating uptake of ribose by OP9 in some way. When either unlabeled bicarbonate or acetate are paired with 13C ribose, the uptake of 13C ribose by OP9 is more distinct. This may be due to *T. auxiliatoris* being stimulated by bicarbonate or acetate to produce that unknown substrate that allows OP9 to grow. However, the other possibility is that OP9 may be stimulated by these substrates, but this is less likely due to its low up take. Overall, further analysis needs to be completed to find a more definitive answer.

Future Directions

Now that it is known that a soluble compound or compounds produced by a variety of sulfate reducers and present in yeast extract are required by OP9, a

logical next step would be to attempt to purify and/or identify the responsible compound(s). One approach would be to fractionate the yeast extract, but this may be difficult due to the complexity of this substrate. An alternative approach would be to find the component(s) common to the supernatants of *T. auxiliatoris* and the other sulfate reducers and test these for their ability to support growth of OP9. However, this is assuming that it is one substrate that allows for growth of OP9, which may or may not be true. Metabolomic approaches along with chemical fractionation could be used to address this question. Additionally, it is not known what fermentation products are produced by OP9, and which of these supports growth of *T. auxiliatoris*. Gas chromatography and high-performance liquid chromatography could be used to determine these substrates.

Another future direction could be to use similar enrichment and isolation procedures employed here to attempt to obtain pure cultures of other members of the Atribacteria from different environments. Also, studies can be made on the composition and possible function of bacterial microcompartments (BMC) in this lineage. Across Atribacteria lineages, BMC is known to be present and the synteny of genes in atribacterial BMC loci are highly conserved (Nobu et al., 2015). Although the substrate that allows OP9 to grow is not specific to *T. auxiliatoris*, both OP9 and SIUC OP9 were partnered with sulfate reducers in their respective co-culture enrichments. This may be a coincidence, but a study to see if these interactions happen in more complex systems may help explain the reason for this observed co-cultivation.

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